Azotobacter Chroococcum- A Plant Growth Promotingrhizobacteria Formulated as Biofertilizer in Vermicast Carrier

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Abstract

Biofertilizers are preparations containing microorganisms efficient strains that help crop plants uptake nutritent and increase soil fertility. Biofertilizer does not pollute the environment and is more beneficial than chemical fertilizers.

Plant Growth Promoting Rhizobacteria (PGPR) are free living, soil borne bacteria, which enhance the plant growth through varies mechanisms like synthesis of growth regulating substances like auxins, cytokinin, and gibberellic acid (GA). In addition, it stimulates rhizospheric microbes, protects the plant from phytopathogens, improves nutrient uptake and ultimately boost up Biological Nitrogen Fixation.The PGPR include genera such as Azotobacter, Azospirillum, Pseudomonas, Burkholderia, Bacillus, and some are members of the Enterobacteriaceae. Azotobacter is a free living nitrogen fixingdiazotroph that has several beneficial effects on the crop growth and yield. The main purpose of this research was to study the plant growth promoting efficiency of rhizobacteria (PGPR) – Azotobacter, when mixed with vermicast carrier material.

The present study focused on isolation of Azotobacter using Ashby's media and Jensen's media which are specific for Azotobacter and Nitrogen fixing bacteria respectively, biochemical identification of Azotobacter, identification of the isolate by 16srDNA sequencing by DNA barcodingtechnique, production of biofertilizer on vermicast carrier and testing the plant growth promoting efficiency of the formulated biofertilizer. Of the several species of Azotobacter, Azotobacterchroococcum happens to be the dominant inhabitant in arable soils capable of fixing nitrogen in culture media. The results confirmed that, the biofertilizerformulated using Azotobacterchroococcum, named as BIOAZOTO-PHS17 can be efficiently used as an alternate for chemical fertilizers.

Keywords
Azotobacter, Biofertilizer, Vermicompost, Plant Growth Promoting Rhizobacteria(PGPR), Biological Nitrogen fixation.

I. INTRODUCTION

Agriculture is derived from Latin words*Ager* and *Cultura*.*Ager* means land or field and *Cultura*means cultivation. The term agriculture means cultivation of land.The science of livestockandproducing crops from the natural resources of the earth.In the past two decades it has been found a wide range of soil bacteria in the rhizosphere can enhance the growth of many important crop plants. Among these bacteria, *Azospirillum*and *Azotobacter* have attracted more attention due to the ability to communicate with important crop plants such as corn, sorghum and wheat [1].

Biofertilizers are preparations containing microorganisms efficient strains that help crop plants uptake nutritent and increase soil fertility.Bio-fertilizers add nutrients through the natural processes of nitrogen fixation solubilizing phosphorus, and stimulating plant growth through the synthesis of growth-promoting substances. Bio-fertilizers can be expected to reduce the use of chemical fertilizers and pesticides[5]. Thebiofertilizeris otherwise known as 'microbial inoculants'. Biofertilizer is defined as a preparation containing latent cells of efficient strains of nitrogen fixing, phosphate solubilizing or cellulytic microorganisms and other rhizosphere microorganisms . Since they play roles, in plant growth is represents "plant-growth promoting rhizobacteria"(PGPR). Therefore, they are enriching soil fertility, plant growth and fulfilling plant nutrient requirements by supplying the nutrients through microorganism and their byproducts.

A) Most Important Microorganisms Usedas Biofertilizer

Organisms that are commonly used as biofertilizers component are nitrogen fixers (N-fixer), potassium solubilizer (K-solubilizer) and phosphate

solubilizer (P- solubilizer), or with the combination of molds or fungi. Several soil bacteria and a few species of fungi possess the ability to bring insoluble phosphate in soil into soluble forms by secreting organic acids [4]. While Rhizobium, Blue Green Algae (BGA) and Azollaare crop specific, bio-inoculants like Azotobacter,Azospirillum, Phosphorus Solubilizing Bacteria (PSB), Vesicular ArbuscularMycorrhiza (VAM) could be regarded as broad spectrum biofertilizers[2][4].The Ksolubilizer, P-solubilizer, free living nitrogen fixing bacteria, are the most important microorganisms used in biofertilizers. Examples for free living nitrogen fixing bacteria, are obligate anaerobes (Clostridiumpasteurianum), obligate aerobes (Azotobacter), facultative anaerobes, photosynthetic bacteria (Rhodobacter), cyanobacteria and some methanogens. The example forKsolubilizer, P-solubilizer, are Bacillus mucilaginous, Bacillus megaterium, Bacillus circulans, BacillussubtilisandPseudomonasstraita.

B) Plant Growth Promoting Rhizobacteria (Pgpr)

Plant Growth Promoting Rhizobacteria (PGPR) represent a wide variety of soil bacteria, and it grown in association with a host plant, result in stimulation of growth of their host. The mode of action of PGPR which act as biofertilizers , either directly by helping to provide nutrient to the host plant, or indirectly by positively influencing root growth and morphology or by aiding other beneficial symbiotic relationships. Not all PGPR are biofertilizers[13] . Many PGPR stimulate the growth of plants by helping to control pathogenic organism of legumes on newly cultivated land required some sort of soilinoculationprocess.

A group of Rhizosphere bacteria (Rhizobacteria) that exerts a beneficial effect on plant growth is referred to as plant growth promoting rhizobacteria (PGPR)[12]. PGPR belong to several genera, e.g.Agrobacterium,Alcaligenes, Arthrobacter, Actinoplanes, Azotobacter, Bacillus, Pseudomonas sp., Rhizobium, Bradyrhizobium, Erwinia, Enterobacter, Amorphosporangium, Cellulomonas, Flavobacterium, Streptomyces and Xanthomonas[14].

The plant growth promoting rhizobacteria (PGPR), are characterized by the following inherent distinctiveness: (i) they must be proficient to colonize the root surface (ii) they must survive, multiply and compete with other microbiota, at least for the time needed to express their plant growth promotion/protection activities, and (iii) they must promote plant growth[10]. About 2–5% of rhizobacteria, when reintroduced by plant inoculation in a soil containing competitive microflora, exert a beneficial effect on plant growth and are termed as plant growth promoting rhizobacteria[10].

C) AzotobacterSp

Azotobacter is a genus of usually motile, oval or spherical bacteria that form thick-walled cysts and may produce large quantities of capsular slime. They are aerobic, free-living soil bacterium which play an important role in the nitrogen cycle in nature, binding atmospheric nitrogeninto the plants, and releasing it in the form of ammonium ions into the soil (nitrogen fixation). In addition, the organism for studying diazotrophs, it is used by humans for the production of biofertilizers. Azotobacterchroococcum was discovered and described in 1901 by the Dutch microbiologist and botanist MartinusBeijerinck.Azotobactersp is sensitive to acidic pH, high salts, and temperature .Azotobacterchroococcum is the most prevalent species found but other species described include A.agilis,A.vinelandii, A. beijerinckii, A.insignis, A.macrocytogenesand A.paspali.

II. MATERIALS AND METHODS

SAMPLE COLLECTION

Rhizosphere soil sample was collected from the paddy field of Nallur near Kurumbur ,Thoothukudi in a sterile polythene bag .

A) Isolation Of Microorganisms From Paddy Field

Soil samples were collected by taking out rhizosphere soil of paddy crop along with roots. The soil was carefully removed from the plant roots and kept in fresh plastic bags after labelling and tagging. These samples were preserved in refrigerator at 4°C temperature for further use . Then the sample was serially diluted . From the dilution 10^{-1}, 10^{-2}, 10^{-3} was taken and spread plated on freshly prepared Ashby's agar plates . Then the plates were incubated at 37^{0}c for 72 hours .

B) ISOLATION Of azotobactersp

Ashby's agar (specific for*Azotobacter*) and jensen's agar (specific for nitrogen free living bacteria) medium was used for the isolation of*Azotobactersp* .

C) Identification And Characterization Of Azotobactersp Morphological Identification

The morphological and biochemical characteristics were compared with those defined in Bergey's manual [10]to confirm them as *Azotobacter*strains. Isolated colony was taken with the help of an inoculating loop and it was uniformly spread over in the centre of a clean microscopic slide. The bacterial cells were fixed by heating it through passing repeatedly few seconds over a flame. They were stained with a drop of crystal violet solution (30

seconds), iodine (30 seconds) followed by ethyl alcohol and safranin (1 to 1.5 minutes) solution. After each staining period, the cells were washed under tap water jet and examined under compound microscope and these bacterial cells were classified as Gram negative (G-) or gram positive (G+) or gram variable (G±) strains[3].The gram negative bacteria will accept the counter stain and becomes pink , while the gram poisitive bacteria remain purple colour. Motility and cyst test also examined in morphological identification. Motility of the isolate was observed using nutrient agar tubes, which differentiate motile and non motilebacteria . For motility testing a fresh 24 hours old broth culture was used . Under sterile condition , a loopful of culture was taken and stab inoculation was done on nutrient deep agar tubes and incubated at 37^0C for 24 hours .The cyst forming ability *Azotobacter* sp was identify by iodine wet mount technique. Cysts of the genus *Azotobacter* are more resistant than the vegetative cells. They are also resistant to drying, ultrasound and gamma and solar irradiation, but not to heating.

D) Biochemical Identification

The biochemical test was performed as catalase activity test. The test shows the ability of some microbes to degrade hydrogen peroxide by producing the enzyme catalase. Usually, all *Azotobacter*species have the capacity to produce oxidases and catalases for the protection of their nitrogenase[6]. Intensive bubbling (*Azotobacter*) was noted as catalase positive, slight bubbling was weakly positive; no gas production was catalase negative. The other biochemical tests are indole test, citrate test, oxidase test, starch hydrolysis, casein hydrolysis, urease test, carbohydrate fermentation of glucose and fructose test, and pigment production and fluorescence test [7] are also carried out to identify the *Azotobacter*sp.

E) DNA Barcoding

In this DNA barcoding, the species of *Azotobacter*is confirmed by using DNA isolation method, polymerase chain reaction(PCR) technique, Agarose gel electrophoresis of PCR product**.**

F) Mass Cultivation Ofazotobactersp

1) Starter Culture-The isolate was inoculated into 100ml of ashby's broth to prepare 1000ml starter culture.

2) Mass Production-For mass production of *Azotobacter*, starter culture is transferred to the 1000 ml of Ashby's broth and continuously agitated for 7 days. When the cell count reached 108- 109 cells/ml, the broth wasused as inoculant. For easy handling, packing, storing and transporting, broth is mixed with vermicompost carrier material which contains sufficient amount of cells.

G) Carrier Parameters

Analysis of physical and chemical properties :The carriers was analyzed for physical and chemical properties including pH, moisture, total nitrogen (Ntot), total phosphorus (Ptot),total potassium (Ktot), electrical conductivity (EC), organic matter, and organic carbon.

H) Formulation Of Carrier Based Inoculum

1 kg of vermicompost carrier material was weighed, sterilized and the temperature was lowered by air drying . The pH was neutralized by adding calcium carbonate . 200 ml of inoculum was mixed with 1 kg of processed vermicompost carrier.The carrier based inoculum was packed in sterile polythene bags, and stored at room temperature and named as BIOAZOTO-PHS17.

I) Quality Control

As per the BIS standard for biofertilizer the count of *Azotobacter* should be 10^8 viable *Azotobacter*cells per gram of the carrier within 15 days of manufacture.Ten grams of BIOAZOTO-PHS17 was taken for estimating viable cells at the initial day, and 10 days after storage using dilution plating method on Ashby's agar and incubated at 37^0C for 3-5 days. After incubation, the colonies were counted and the total viable cells were calculated.

J) Determining The Plant Growth Promoting Ability Ofbioazoto-Phs17

To determine the plant growth promoting ability of BIOAZOTO-PHS17,it was applied to tomato seeds[13].BIOAZOTOPHS 17 pot was filled with garden soil and BIOAZOTO-PHS17, Control pot 1 was filled with garden soil and vermicompost , Control pot 2 was filled with garden soil .After that tomato seeds were sowed in all the three pots and placed outdoor to provide a proper light and air conditions. Growth of the plants were continuously monitored.

III. RESULTS AND DISCUSSION

In this present work, the Plant Growth Promoting Rhizobacteria (PGPR) – *Azotobacter*sp was isolated, identified, characterized and formulated into a carrier based inoculum in a vermicast carrier and its plant growth promoting efficiency was determined.

The collected rhizosphere soil sample was serially diluted up to10^{-7} and the dilutions 10^{-1}, 10^{-2}, 10^{-3} was spread plated on to the Ashby's agar plates. Incubated at 37^0c for 72 hours.Based on colony morphology the isolated colonies were streaked onto the selective agar medium such as Ashby's agar (specific for*Azotobacter*) and Jensen's agar (specific

for nitrogen free living bacteria) medium . Incubated at 37^0c for 72 hours .

In Ashby's agar plates, the colonies were large, flat, soft, milky, mucoid, and , gummy growth indicated that the isolated organism may be *Azotobacter*sp.In Jensen's agar plates, the colonies were raised, spherical flat and few with irregular margins .

Various tests such as morphological, biochemical and genetic identification were carried out to identify the isolate. The morphological identification methods were gram staining and motility test. The biochemical identification methods were Indole test, Citrate test, Catalase test, Oxidase test, Starch hydrolysis, Casein hydrolysis, Urease test, Carbohydrate fermentation. 16 s rDNA sequencing technique was adopted for genus and species level identification of the isolate.The isolate was identified as gram negative, oval or rod shaped bacterium, aerobic, cyst forming bacteria. It can utilize citrate as their carbon source. It can produce an enzyme catalase that breakdown hydrogen peroxide to water and oxygen, can reduce nitrate and hydrolyse starch, casein .

Pigment are also an important characteristic and produced by all *Azotobacter*sp . After 7 days of incubation, the colonies produced pigment .The isolate *Azotobacter*spwas further subjected togenomic DNAand phylogenetic analysis**,** which included the sequencing of its 16srDNA and exploring similarly search with genBANK DATA using basis logic alignment search tool (BLAST).The resulting DNA fragments were visualized using an ultraviolet Transilluminator.The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Neimodel . The tree with the highest log likelihood (-745.45) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 515 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. (Table I)

Physical and chemical properties of carriers was analyzed. The parameters of pH, moisture, total nitrogen (Ntot), total phosphorus (Ptot) and total potassium (Ktot), electrical conductivity (EC), organic matter, organic carbon.(Table II)

BIOAZOTO PHS 17 pot , Control pot 1, Control pot 2 was sowed with tomato seeds , and it was continuously monitored . Plant height , root length , leaf length , seed germination of the plants control pot 1 and pot 2 and BIOAZOTO-PHS 17 under study was measured upto 2-3 weeks .(Fig 2,3,4,5,6)

The BIOAZOTO-PHS 17 which was supplemented with *Azotobacter*showed increased growth than the control pot 1 and pot 2.This shows that *Azotobacter* has plant growth promoting activity.(Fig 7)

In this present study carrier based inoculum of *Azotobacterchroococum* was named as BIOAZOTO - PHS 17. The plant growth promoting ability of BIOAZOTO – PHS17 was determined and it showed better result in promoting plant growth in tomato.*Azotobacter*improves seed germination, plant height, root length, shoot length. It helps to increase nutrient availability and to restore soil fertility for better crop response.

Table I Phylogenetic identification of Azotobacterchroococcum

Table II Physiochemical analysis of vermicast

CARRIER - VERMICOMPOST			RESULT CARRIER VALUE	GENERAL CARRIER VALUE		
	Parameters		resultvalue	Low	Medium	High
1	pH	Acidic	6.78	6.0-7.0	4.0-6.0	2.5-4.0
		Alkaline		6.0-7.0	7.0-9.0	Above 9.0
2	EC		0.51	1.0	1 to 2	2 to 4
3	Organiccarbon(%)		0.60	0.86	0.86-12.9	Above 12.9
4	Organic matter		1.032			
5	Available nitrogen (kg/ha)		134.4	280.0	280.0-560.0	Above 560
6	Available phosphorus kg/ha		23.74	10.0	10.0-25.0	Above 25.0
7	Available potassium (kg/ha)		460	110.0	110.0-280.0	Above 280.0

Table III Determining of initial viable count of *Azotobacterchroococcum* in BIOAZOTO – PHS 17

S.NO	MEDIA	DILUTIONS	NO.OF COLONIES
1	Ashby's agar medium	10^{-1}	TNTC
2		10^{-2}	TNTC
3		10^{-3}	262
4		10^{-4}	193
5		10^{-5}	166
6		10^{-6}	130
7		10^{-7}	108

Table IV Determining of viable count of *Azotobacterchroococcum* in BIOAZOTO – PHS 17 after 10 days

S.NO	MEDIA	DILUTIONS	NO.OF COLONIES
1	Ashby's agar medium	10^{-1}	TNTC
2		10^{-2}	TNTC
3		10^{-3}	262
4		10^{-4}	193
5		10^{-5}	166
6		10^{-6}	130
7		10^{-7}	108

Fig 1 Determining of initial and 10 days viable count of *Azotobacterchroococcum* in BIOAZOTO – PHS 17 .

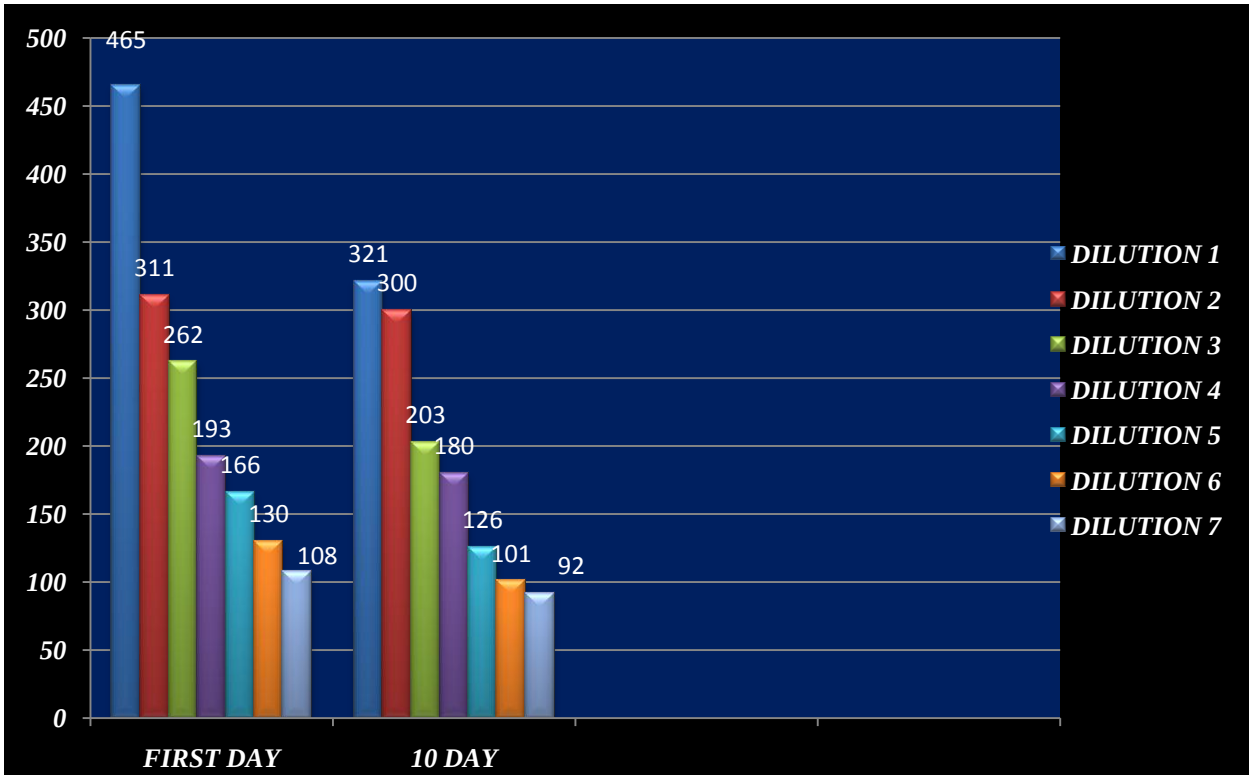


Fig 2 Determining the plant height of plant growth promoting ability of BIOAZOTO – PHS 17

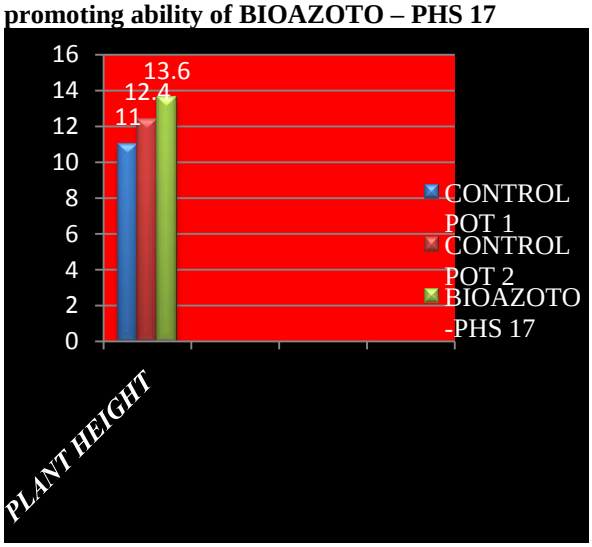
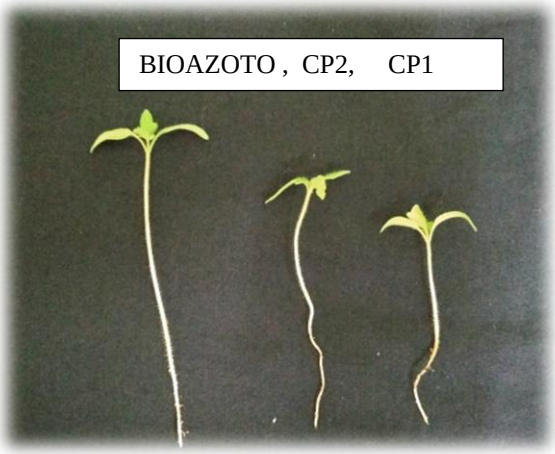


Fig 3Determining the leaf length of plant growth promoting ability of BIOAZOTO – PHS 17



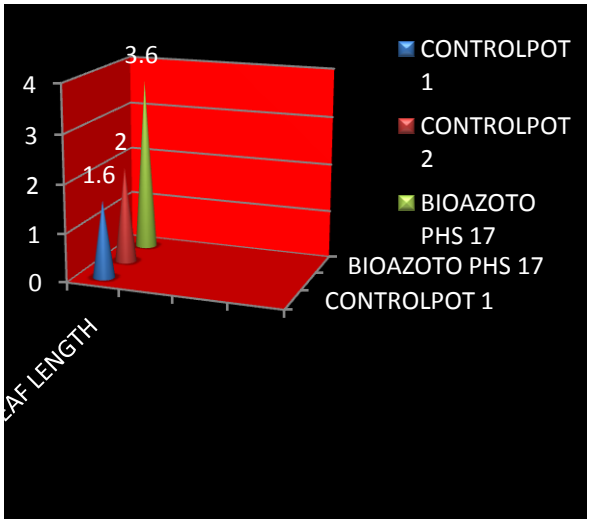


Fig 4 Determining the root length of plant growth promoting ability of BIOAZOTO – PHS 17

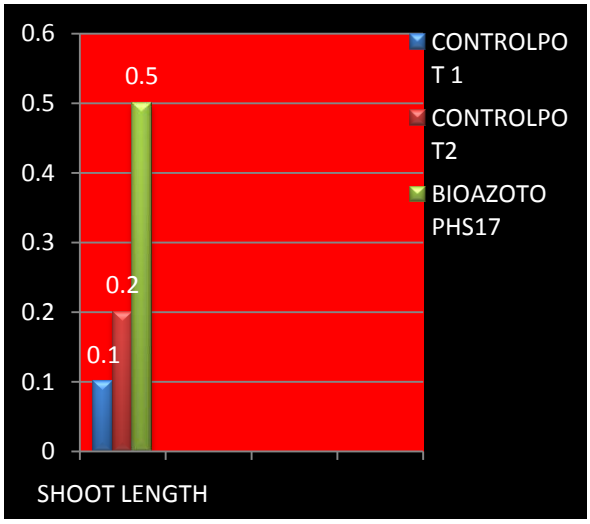


Fig 6 Determining the seed germination of plant growth promoting ability of BIOAZOTO – PHS 17

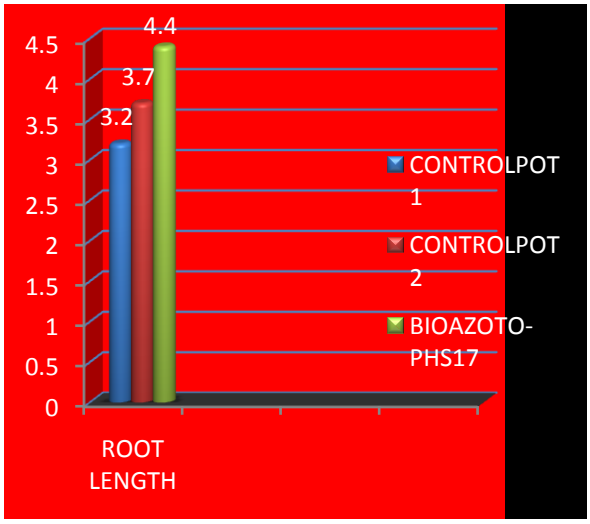
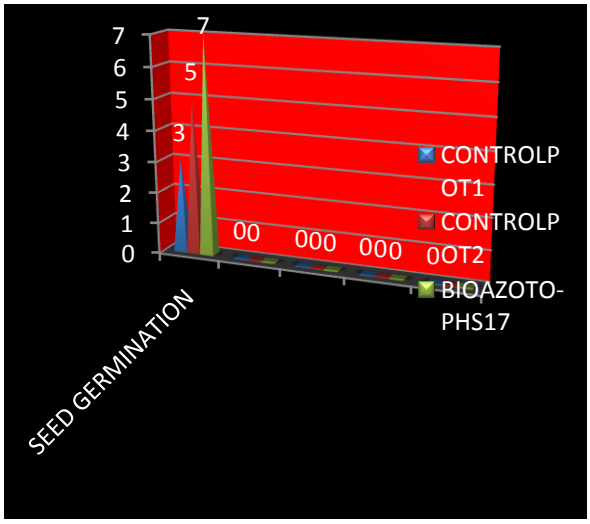


Fig 3.aDetermining the leaf length of plant growth promoting ability of BIOAZOTO – PHS 17



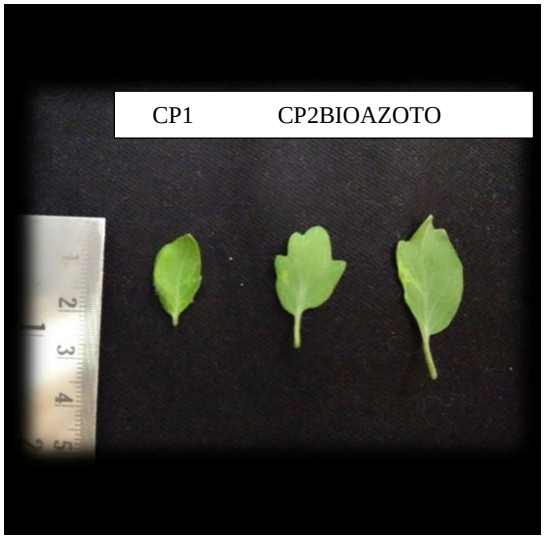


Fig 5 Determining the shoot length of plant growth promoting ability of BIOAZOTO – PHS 17

IV. CONCLUSION

Hereby I conclude from this study that Azotobacterchroococum a PGPR can be efficiently used as a biofertilizer for tomato plant when applied as a carrier based inoculum. The use of BIOAZOTO – PHS17 – a biofertilizer formulated in this present study will be a solution for an ecofriendly environment by reducing the use of chemical fertilizers and thereby preventing soil and ground water pollution and will be a boon for farmers to have an organic agriculture and to increase crop yield in agricultural crops.

Fig7 Comparison of plant growth between BIOAZOTO PHS 17 and control pots

REFERENCE

[1] Afify A H, Aida H and Nassef M A (2003). Yield and nitrogen assimilation of winter wheat inoculated with new recombinant inoculants of rhizobacteria. Biological Sciences. 6: 344-358

[2] Biofertilizer a novel tool for agriculture Boraste.A, Vamsi.K.K, Jhadav.A, Gupta.M, Joshi.B, Gupta.N, Gupta.G

[3] Cerney, G. (1993). Method for the distinction of gram negative and gram positive bacteria. *EU J. Appl Micro*. (3), 223-225.

[4] Gupta A.K. 2004. The complete technology book on biofertilizers and organic farming. National Institute of Industrial Research Press. India.

[5] In vitro studies on the effect of biofertilizer (Azotobacteria and rhizobium on seed germination and develop of Trigonellafoenumgraecum. L using a novel glass morble containing a liquid medium by G.S Naganandha,arijitdas ,SouravBhattacharta and T.Kalpana

[6] Jimenez, J.D., Montana, J.S., Martínez, M.M. (2011). Characterization of free nitrogen fixing bacteria of the genus *Azotobacter*in organic vegetable-grown Colombian soils. *Braz. J. Microbiol.* 42(3) 66-78.

[7] Johnstone, D. B. **(1955).** Azotobacter fluorescence. J. ***Bact.*** 69, **481.**

[8] Kloepper, 1994 J.W. KloepperPlant growth-promoting rhizobacteria (other systems)Y. Okon (Ed.), *Azospirillum*/Plant Associations, CRC Press, Boca Raton, FL, USA (1994), pp. 111-118

[9] Kloepper, J.W., Schroth, M.N., 1978. Plant growth-promoting rhizobacteria on radishes. In: Proceedings of the 4th International Conference on Plant Pathogenic Bacteria, vol. 2. Station de PathologieVégétale et de Phytobactériologie, INRA, Angers, France, pp. 879–882

[10] Krieg N.R., Holt J.G., Sneath P.H.A., Staley J.T., and Williams S.T. (1994). Bergey's Manual of Determinative Bacteriology (9th ed.), Williams & Wilkins, Baltimore, Md, USA

[11] Martyniuk, S., and Martyniuk. M., (2003). Occurrence of *Azotobacter Sp*. in some polish soils. *Polish journal of environmental studies*, **12,** p: 371-374.

[12] Schroth M.N and Hancock J.G. 1981. Selected topics in biological control. Ann Rev Microbiol. 35: 453-476.

[13] Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255, 571–586

[14] Weller D.M. 1988. Biological control of soil borne plant pathogens in the rhizosphere with bacteria. Ann Rev Phytopathol. 26: 379-407.

[15] Zaied KA, Abd El-Hady AH, Afify-Aida H, et al. Pakistan Journal of Biological Sciences 2003, 6(4):344-358.